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Report

Marine Viruses Exploit Their Host's Two-Component Regulatory System in Response to Resource Limitation

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Summary

Phosphorus (P) availability, which often limits productivity in marine ecosystems, shapes the P-acquisition gene content of the marine cyanobacteria *Prochlorococcus* [1–4] and its viruses (cyanophages) [5, 6]. As in other bacteria, in *Prochlorococcus* these genes are regulated by the PhoR/PhoB two-component regulatory system that is used to sense and respond to P availability and is typical of signal transduction systems found in diverse organisms [7]. Replication of cyanophage genomes requires a significant amount of P, and therefore these phages could gain a fitness advantage by influencing host P acquisition in P-limited environments. Here we show that the transcription of a phage-encoded high-affinity phosphate-binding protein gene (*pstS*) and alkaline phosphatase gene (*phoA*)—both of which have host orthologs—is elevated when the phages are infecting host cells that are P starved, relative to P-replete control cells. We further show that the phage versions of these genes are regulated by the host's PhoR/PhoB system. This not only extends this fundamental signaling mechanism to viruses but is also the first example of regulation of lytic phage genes by nutrient limitation in the host. As such, it reveals an important new dimension of the intimate coevolution of phage, host, and environment in the world's oceans.

Results and Discussion

Unicellular cyanobacteria *Prochlorococcus* and *Synechococcus* are the dominant photosynthetic organisms in the oceans and they contribute significantly to global primary production [8]. The environmental availability of phosphorus (P), often a limiting nutrient in marine ecosystems, exerts strong selective pressure on *Prochlorococcus* genomes, which is manifested in the suite of P-acquisition genes they contain [1–4]. This selective pressure is also visible in cyanophages (viruses that infect cyanobacteria): 9 of the 16 sequenced T4-like cyanophages isolated on *Prochlorococcus* and *Synechococcus* [9] contain *pstS*, encoding a periplasmic high-affinity phosphate-binding protein, and 2 contain *phoA*, an alkaline phosphatase gene. We have suggested previously that these genes of host origin play a role in the acquisition of phosphorus required for phage DNA replication [6]. Consistent with this hypothesis, the frequency of occurrence of *pstS* gene in phages in the wild was shown to be higher in oceanic sites with lower phosphate [5], as is true of their *Prochlorococcus* hosts [4, 10].

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Both *pstS* and *phoA* are upregulated in *Prochlorococcus* [2] and *Synechococcus* [11] during P starvation, a signal transduction response regulated by the bacterial phosphate sensing two-component system. We postulated that the expression of the phage versions of these P-acquisition genes (hereafter referred to as "phage *pstS*" and "phage *phoA*") might be regulated by P availability to the hosts—possibly through the PhoR/PhoB two-component regulatory system in the host cells. To address this question, we used a cultured *Prochlorococcus* strain (NATL2A) and the T4-like cyanophage P-SSM2, which encodes *pstS*, to measure expression of selected phage and host genes, under P-depleted and P-replete conditions (Figure 1). Transcript levels of the host *pstS* gene increased within 8 hr in the P-limited host cells (Figure 1B) and rose steadily thereafter, signaling the onset of P starvation, which was manifested as a decrease in the culture growth rate (Figure 1A). Reintroduction of phosphate at 46 hr (arrow, Figure 1B) resulted in a rapid decline of *pstS* transcripts as the cells were released from P starvation. Similar patterns have been observed in other strains of *Prochlorococcus* under P starvation [2].

We used this system to examine the effect of host P starvation on infection kinetics and *pstS* expression level in infecting phage. The cultures were infected 47 hr after resuspension in P-depleted media (shaded interval in Figure 1B) when P starvation was well established in the host cells, as indicated by upregulation of *pstS* (Figure 1B). Both host and phage *pstS* transcript levels were then measured over time. Host *pstS* transcript levels in infected P-starved cells stayed higher than those in the P-replete cultures (Figure S1A available online), as was observed in uninfected hosts (Figure 1B). Transcript levels of phage *pstS* increased in P-starved hosts relative to the P-replete control (Figures 1C and S1B), whereas transcripts of genes *g61* and *g20*—early and late T4-like phage structural genes [12–14], respectively—did not (Figures 1C and S1B), suggesting differential regulation of *pstS* and phage structural genes. Addition of phosphate 4 hr after infection (arrow, Figure 1C) resulted in a reduction of both host (similar to Figure 1B) and phage *pstS* transcripts within 4 hr (Figure 1C), suggesting that both phage and host *pstS* genes could be regulated by the same mechanism.

phoH encodes an ATP binding protein with unknown function [15] and is considered a phosphate (*pho*) regulon gene because it is upregulated by P starvation in *E. coli* [16, 17]. Its expression is not upregulated during P starvation in marine cyanobacteria [2], however, suggesting that it may not play the same role as in *E. coli* [18]. Nonetheless, because of its prevalence in T4-like cyanophages [9] and association with the *pho* regulon in *E. coli*, we examined its expression in our experiments. We found that the expression of *phoH* in the phage (Figures 1C and S1B) and host (Figure S1A) was not affected by P starvation, and therefore its role in both host and phage remains a mystery.

That the level of P starvation of the host cell selectively influences the degree of upregulation of phage *pstS* suggests coevolution of regulatory systems between host and phage—probably involving the PhoR/PhoB two-component regulatory system widely used by bacteria including

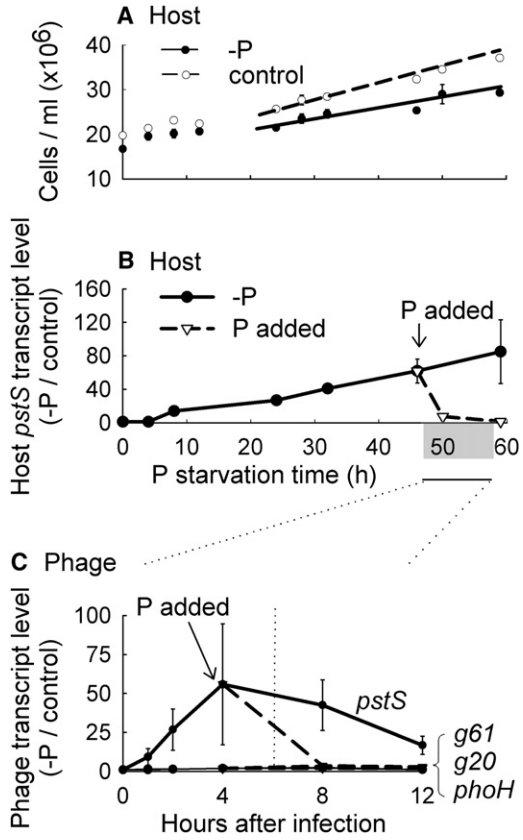

Figure 1. Host and Phage Transcript Levels during Infection of *Prochlorococcus* NATL2A by Cyanophage P-SSM2 Under P-Depleted and P-Replete Conditions

(A and B) Uninfected host cells. P starvation was achieved by resuspending log-phase cells in P-depleted (-P) media at t = 0 hr and letting the culture grow.

(A) Cell number as a function of time in P-replete control cultures (open circles) compared to cells resuspended in media with no amended P at t = 0 hr (-P, closed circles).

(B) Host *pstS* (YP_291636.1) transcript levels in uninfected cells during P starvation relative to P-replete controls. The arrow indicates the reintroduction of phosphate at 46 hr to verify the P-starvation condition.

(C) Phage gene expression in infected cells. Aliquots from the cultures in (A) were infected with phage 47 hr after the onset of P starvation and infection was then monitored for 12 hr (shaded interval shown in B). Transcript levels of phage genes *pstS*, *phoH*, *g61* (DNA primase), and *g20* (portal protein) were measured during infection of P-depleted host cells and normalized to their levels when infecting P-replete cells. The only transcript levels that differed from the controls was that of *pstS*, and therefore all others are flat lines near 1 on the *y* axis. The arrow indicates reintroduction of phosphate 4 hr after infection and dashed lines indicate transcript levels after phosphate reintroduction. Error bars indicate the SD of two biological replicates and are smaller than the data point when not apparent. The vertical dotted line indicates the beginning of phage burst, as determined in Figure S1F.

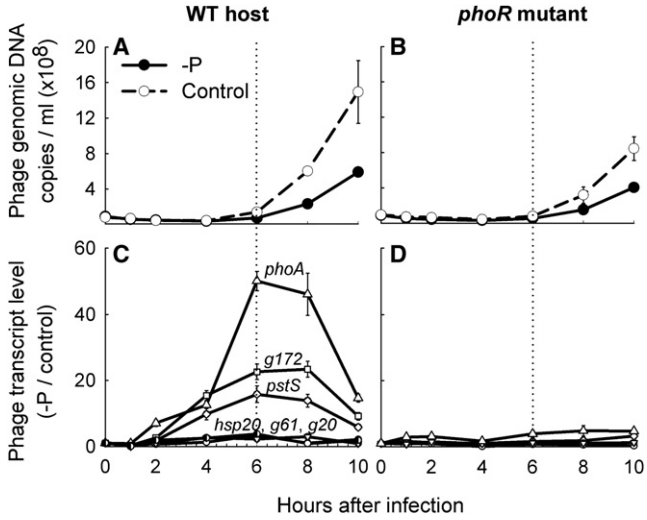

Figure 2. Infection and Gene Expression Patterns in Cyanophage S-SM1 Infecting a Wild-Type and Mutant Strain of *Synechococcus* WH8102 under P-Depleted and P-Replete Conditions

(A and B) Phage genome copies released while infecting P-replete (control) and P-depleted (-P) WT (A) and mutant (B) strains. The WT and mutant strains were transferred to P-replete (control) and P-depleted (-P) media, and once P limitation was established in the -P cultures, as evidenced by upregulation of *pstS* in the WT strain (not shown), they were infected by phage at t = 0 hr. The vertical dotted line indicates the beginning of phage burst.

(C and D) Transcript levels of phage genes in the P-depleted host infections relative to P-replete controls: *pstS*, *phoA*, *g172*, *hsp20*, *g61*, and *g20*. Error bars indicate the SD of two biological replicates.

cyanobacteria during P starvation [19, 20]. The obvious way to test this hypothesis would be to measure phage *pstS* expression in a mutant host without the PhoR/PhoB system. Because there is no genetic system for *Prochlorococcus*, we used a *phoR* knockout mutant of its close relative, *Synechococcus* WH8102 [11], and the T4-like cyanophage S-SM1 [9], which contains *pstS* (and also *phoA*).

We first established that upon the onset of P starvation in host cells, *pstS* was not upregulated in the *phoR* mutant, although it was in the WT cells—simply confirming the results of Tetu et al. [11] (data not shown). Once *pstS* was upregulated in the P-starved WT host, we infected P-starved and control cultures of both the mutant and WT strains with phage and measured phage production and the expression levels of a suite of phage genes over the course of infection (Figure 2). Phage production in P-starved cells was reduced relative to the P-replete controls for both the WT and *phoR* mutant (Figures 2A and 2B), confirming that the mutant was indeed P starved and agreeing with our results for *Prochlorococcus* phages (Figure S1F). The *phoR* mutant (doubling time 1.43 ± 0.04 days) grew slower than the WT (1.39 ± 0.04 days) in P-replete conditions, which could explain why phage production was lower in the *phoR* mutant than in the WT host (Figures 2A and 2B). Most importantly, phage *pstS* and *phoA* transcripts increased relative to controls in P-starved WT host cells (Figure 2C), as in our *Prochlorococcus* phage/host system (Figure 1C), but they did not in the mutant lacking the PhoR/PhoB regulatory system (Figure 2D). As before, and as expected, transcript levels of early and late phage structural genes *g61* and *g20*, respectively, were not affected by P starvation of the WT or mutant cells (Figures 2C and 2D). The significance of the expression patterns of genes *g172* and *hsp20* in this experiment will become clear below.

The evidence presented thus far is compelling that the transcription of the phage *pstS* (and *phoA*) is regulated by the host PhoR/PhoB two-component system. What is the mechanism? Host genes regulated by the PhoR/PhoB system (*pho* regulon genes) have well-conserved *pho* box sequences in their promoters, which bind the transcriptional activator PhoB [16]. *pho* box sequences, which contain conserved

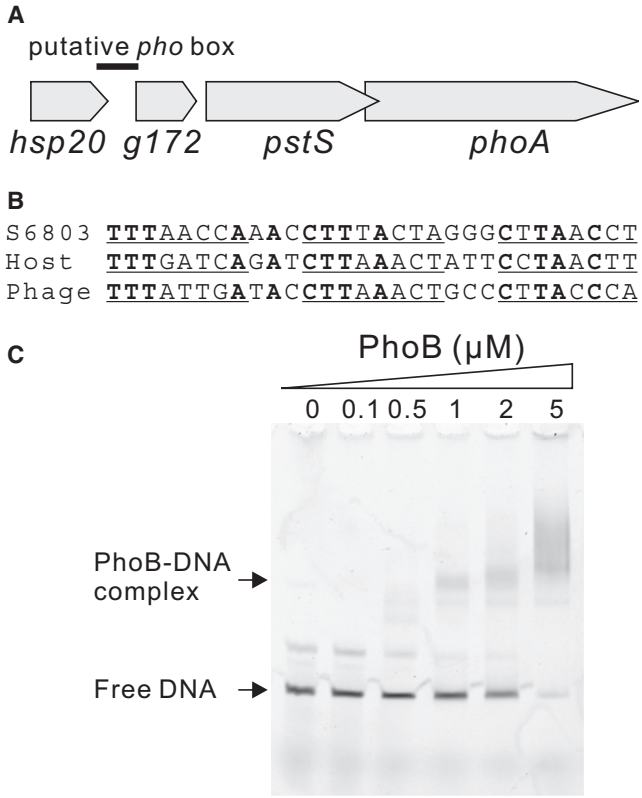

Figure 3. Binding of Recombinant *Synechococcus* WH8102 PhoB to the Upstream Region of *pstS* in Phage S-SM1

(A) Genomic organization of the S-SM1 *pstS* region, showing *hsp20*, *g172*, *pstS*, and *phoA*. A black bar indicates the ~200 bp DNA fragment containing the putative *pho* box that was used in the binding assays shown in (C).
(B) Alignment of the experimentally determined *pho* box sequence of *Synechocystis* sp. PCC6803 (S6803) [19] and the putative host and phage *pho* box sequences. The 8 bp conserved tandem repeats are underlined, which are important for PhoB binding. Bold letters indicate conserved sequences.
(C) DNA fragment containing the putative *pho* box was labeled by a fluorophore and incubated with increasing concentrations of purified recombinant *Synechococcus* WH8102 PhoB protein. Free DNA and the shifted PhoB-DNA complex were separated in native polyacrylamide gels.

tandem 8 bp repeats—important for PhoB binding [21]—separated by flexible 3 bp linkers [16], have been experimentally determined in *Synechocystis* sp. PCC6803 [19]. They have also been predicted in *Prochlorococcus*, *Synechococcus* [22], and their phages [9]. Thus we hypothesized that the likely mechanism for the regulation of these phage genes via the host *pho* regulon would be through binding of host PhoB to the promoters of the phage genes—activating their transcription.

In cyanophage S-SM1, *pstS* is upstream of *phoA*, with which it overlaps. Just upstream of *pstS* are *g172* and *hsp20* (Figure 3A). When the phage infects a P-starved WT *Synechococcus* host cell, *g172*, *pstS*, and *phoA* are upregulated in the phage (Figure 2C), whereas *hsp20* is not (Figure 2C). This suggests that *g172*, *pstS*, and *phoA* are transcribed from the same promoter—probably located between *g172* and *hsp20* (black bar, Figure 3A)—which is activated by binding of the host PhoB protein to a *pho* box. To explore this further, we searched for (by manual alignment) and found a putative *pho* box sequence in this region (Figures 3B and S2A) with three tandem 8 bp sequences 70.8% identical to the predicted host *Synechococcus* WH8102 *pho* box [22]. We purified recombinant *Synechococcus* WH8102 PhoB protein (see Experimental Procedures) and PCR amplified a ~200 bp DNA fragment upstream of *g172* (Figure 3A) containing this putative *pho* box and used it in binding assays. A gel mobility shift assay showed that the recombinant *Synechococcus* WH8102 PhoB protein binds to this DNA fragment (Figure 3C) and that this binding reaction is sequence specific (Figure S2B), suggesting that this putative *pho* box is functional. Phage gene *g172*, which is not found in any host genomes, is between the putative *pho* box and the phage *pstS* gene (Figure 3A). The putative *pho* box sequence in front of *g172* could be formed or gained after *g172* and *pstS* got inserted in the phage genome. Or, this putative *pho* box could be gained together with *pstS* from the host genome and *g172* was inserted between them during gene recombination events, which could destroy the *pho* box. In both scenarios, there must be strong selective pressure to maintain this putative *pho* box in front of *pstS* in the phage, even though it need not be in that location in the host genome. Thus, cyanophages not only carry *pstS* and *phoA* genes, but the expression of these genes is tied into the host PhoR/PhoB phosphate-sensing two-component regulatory system, which regulates the P-starvation response of the host. Although we can't completely exclude the possibility of indirect regulation of these phage genes by the PhoR/PhoB system, the results of our binding assay strongly favors direct regulation.

Regulation of phage *pstS* and *phoA* genes by P availability to the host also suggests that these phage genes play a role in the acquisition of phosphorus for phage DNA replication during infection. For *Prochlorococcus* (Figure S1F) and *Synechococcus* (Figures 2A and 2B) infecting phages with the *pstS* gene, we found that phage production was reduced by P starvation (Figures S1F, 2A, and 2B), but the timing of the infective cycle was not changed (Figures S1C–S1F). In a related cyanophage/host system, in which the phage does not carry the *pstS* gene, the lytic cycle is lengthened during infection of P-starved host cells [23], consistent with our hypothesis that phage *pstS* expression may augment the P supply to the host cell to facilitate an expedient lytic cycle. Phage-encoded *phoA* may facilitate access to organic P pools, which again would give phage a selective advantage in phosphate-limited environments. Cyanophage S-SM1 gene *g172* may play a role in this process as indicated by the fact that its expression is also induced by P starvation, although its function is still unknown. Because of the lack of genetic tools, we couldn't knock out these cyanophage genes to see how they affect the lytic cycle, although the selective advantage for cyanophages to have *pstS* gene is supported by the fact that it is enriched in phage genome fragments at oceanic sites with lower phosphate concentrations [5].

Two-component regulatory systems have been found in the three kingdoms of life [7] enabling cells to better acclimate to changing environmental conditions. Although nutrient limitation has been shown to affect the lysis-versus-lysogenization decision of coliphage λ through the host ppGpp level [24, 25], to our knowledge this is the first example in which a lytic virus exploits a host two-component system to be responsive to "environmental conditions" within the host cell, which in turn is responding to nutrient limitation in the external milieu. This extends the selection pressures on the host cell to its infecting viruses, making ever more intimate the coevolution of viruses, hosts, and their environment.

Experimental Procedures

Strains and Growth Conditions

Axenic *Prochlorococcus* NATL2A was grown in 0.2 μm filtered Sargasso seawater-based Pro99 medium [26] amended with 10 mM HEPES (pH 7.5) and 12 mM sodium bicarbonate. Axenic *Synechococcus* WH8102 was grown in SN medium [27] made with seawater from Woods Hole, MA. Kanamycin (25 μg ml^{-1}) was used to maintain the *phoR* mutant [11]. Cultures were maintained at 21°C under constant cool white light (30 μmol quanta m^{-2} s^{-1}).

Prior to infection, the NATL2A culture was harvested by centrifugation (15,000 × g for 10 min), washed twice in either PO_4-replete (Pro99 with 50 μM PO_4) or -depleted (Pro99 without PO_4) medium, and resuspended in the same medium. WH8102 and the *phoR* mutant cultures were transferred 1:25 to PO_4-replete or -depleted SN medium. Cyanophage P-SSM2 lysate was concentrated by centrifugation (5,000 × g) with Amicon Ultra-15 30K Centrifugal Filter Units (Millipore), washed twice in filtered Sargasso seawater, and resuspended in the same medium. Cyanophage S-SM1 lysate was concentrated the same way and resuspended in filtered seawater from Woods Hole. Infection was carried on with a multiplicity of infection of 3. Total cell concentration was determined by flow cytometry (Influx, Cytopeia-BD), and phage concentration was determined by the most probable number assay [28].

Quantification of Phage and Host Genomic DNA during Infection

Phage and host genomic DNA was quantified with a quantitative PCR method described previously [29]. In brief, infected *Prochlorococcus* cells were filtered through polycarbonate filters (0.2 μm pore-size) to separate extracellular phage (filtrate), from host cells containing both host and intracellular phage genomic DNA. The latter were recovered from the filters. The qPCR primers used to measure DNA in the filter and filtrate fractions are listed in Table S1.

RNA Extraction and Transcript Analysis

Samples were collected by centrifugation at 15,000 × g for 15 min at 4°C, and cell pellet was flash frozen in liquid nitrogen and stored at −80°C. Total RNA was then extracted with the Ambion mirVana RNA isolation kit and DNA was removed with the Turbo DNA-free kit (Ambion). Total RNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). cDNA copies were quantified with a QuantiTect SYBR Green PCR Kit (QIAGEN) with 0.5 μM forward and reverse primers (Table S1) on a LightCycler 480 Real-Time PCR System (Roche Diagnostics). The qPCR primers were designed to amplify only the gene of interest. The specificity of phage (or host) *pstS* gene primers was confirmed by showing that they don't amplify the host (or phage) *pstS* genes (data not shown). The amplification reaction consisted of an initial activation step of 15 min at 95°C, then 50 cycles of denaturation (95°C, 15 s), annealing (56°C, 30 s), and extension (72°C, 30 s), followed by 5 min at 72°C. Relative transcript abundance was determined by the ΔΔCT method [30]. The host *rnpB* gene was used as an internal control for our gene expression study, as shown by the fact that transcription of this gene is stable in various conditions, including phage infection [29] (Figure S3).

Protein Expression and Purification

Primers 8102phoBNcoI (5′-AAAAAACCATGGCTATGCCCGCCGCTGTGGC-3′) and 8102phoBHindIII (5′-TTTTTTAAGCTTCAGCCGAACCGGTAGCCG-3′) were used to amplify the *Synechococcus* WH8102 *phoB* gene. PCR was carried out with 0.02 U/μl KOD HiFi DNA polymerase (Novagen) in 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.001% BSA, 1 mM $MgCl_2$, 0.2 mM dNTPs, and 0.4 μM of each primer. PCR cycling conditions consisted of a hot start at 94°C for 5 min, followed by 25 cycles (98°C for 15 s, 50°C for 2 s, and 74°C for 20 s), followed by incubation at 74°C for 7 min. This gene was then cloned into pET30a plasmid (Novagen) with a His-tag on the 5′ end and transformed into *E. coli* BL21(DE3) competent cells. A single colony was grown at 37°C overnight in LB medium containing 50 μg/ml Kanamycin, diluted 1:100 with the same medium, and grown at 37°C until OD_{600} = 0.5. Protein expression was induced by adding IPTG to a final concentration of 0.1 mM and cells were grown at 18°C for 24 hr. Cells were harvested by centrifugation at 6,000 × g for 20 min. The cell pellet was suspended with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole [pH 7.4]) and disrupted by sonication. The crude lysate was centrifuged at 10,000 × g for 20 min and the supernatant was loaded on a HisTrap FF crude column (GE Healthcare). Protein purification was performed according to the manufacturer's instructions. Protein was eluted from the column with elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole [pH 7.4]). Imidazole was removed from the protein solution by centrifugation (5,000 × g, 15 min) with Amicon Ultra-15 10K Centrifugal Filter Units (Millipore), washed twice with 20 mM NaH_2PO_4 (pH 7.4), and resuspended in the same buffer. Although PhoB needs to be phosphorylated by PhoR to become active, the DNA binding ability of purified recombinant PhoB from *E. coli* is comparable to that of phosphorylated PhoB [20].

Electrophoretic Mobility Shift Assay

Primers SSM1F187bp (5′-ATGGAATGCTGACAATTAATTTACAA-3′) and SSM1R10_6FAM (5′-6FAM-CTTTTTTCATTTCTTTGTATGTGTGAA-3′) were used to amplify a 200 bp fragment upstream of phage S-SM1 *g172*. The reverse primer SSM1R10_6FAM was 5′ labeled with the fluorophore 6FAM to enable in-gel detection with a fluorescence scanner. The same primers without labeling were used to amplify the nonlabeled specific competitor DNA fragment. Nonspecific competitor DNA fragment was amplified with primers SSM1F357bp (5′-TAAAGACGAAATCGAAGTGAGCAC-3′) and SSM1F187bp_RC (5′-TTGTAAATTAATTGTCAGCATTCCAT-3′). PCR products were purified with QIAquick PCR Purification Kit (QIAGEN). For binding assays without competitor, 3 nM labeled DNA fragment was incubated on ice for 30 min with different amounts of PhoB protein in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 2.5% glycerol, 5 μg/ml poly-dIdC, 2 μg/ml BSA, and 1 mM DTT in a 20 μl reaction. For assay with competitors, 3 nM labeled DNA fragment and 1 μM PhoB protein were incubated with different amounts of nonlabeled specific competitor or nonspecific competitor. The free DNA and protein-bound complexes were separated on 5% native polyacrylamide gel with 1× TBE buffer (89 mM Tris, 2 mM EDTA, 89 mM Boric acid [pH 8.3]).

Supplemental Information

Supplemental Information includes three figures and one table and can be found with this article online at doi:10.1016/j.cub.2011.11.055.

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